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(54) Title: NUCLEIC ACID CHELATE CONJUGATE AS THERAPEUTIC AND DIAGNOSTIC AGENTS

(57) Abstract

Novel conjugates are provided comprising, in particular, stable nucleotide sequences joined to a chelating agent. The conjugates find use in inhibiting expression of mRNA, in killing pathogens, and in providing selection for particular classes of cells or killing particular classes of cells. Polyamine, polycarboxylic acid, chelating agents are employed in the conjugates as exemplary.

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NUCLEIC ACID CHELATE CONJUGATE AS THERAPEUTIC AND DIAGNOSTIC AGENTS

INTRODUCTION

Technical Field

The field concerns the use of modified oligonucleotides as therapeutic agents, inhibiting maturation or expression of transcription products *in vivo* and *in vitro*.

Background

The biological revolution has introduced a variety of new techniques resulting in the ability to determine various cellular and subcellular processes. As the understanding has increased as to how the cell maintains its viability and proliferates, new opportunities have opened for utilizing novel therapeutic approaches. One technique which has been used in a variety of ways in the laboratory and is being expanded from the laboratory into real life situations, is the use of anti-sense sequences to modulate the fate of a transcription product in a host cell.

For the most part, the approaches in employing anti-sense sequences have been twofold. In one approach, cells in culture are modified by injection of a large excess of a DNA sequence which is complementary to the sequence of a mRNA present in the cell. A substantial reduction in the expression product is observed. In another approach, one can introduce a transcription cassette comprising a promoter functional in the host and a DNA sequence which results in the production of a mRNA which is complementary to an endogenous mRNA. Again, one observes a reduction in the expression product to which the transcription construct is directed.

If, however, anti-sense sequences are to become a useful therapeutic agent, there are many problems and difficulties to be overcome. As a therapeutic agent, a method must be found which allows for the transfer of the anti-sense sequence across the cellular membrane. The anti-sense sequence must be designed so as to be relatively stable to degradation, particularly by nucleases. In addition, there remain concerns about the specificity of the sequence, particularly where one does not wish to kill the host cell. Also of concern is how to reach the necessary concentration in the cell to provide the desired level of inhibition of transcription product maturation or expression. Finally, any technique which is devised for fulfilling the above objectives, must take into consideration such problems as toxicity, immunogenicity, solubility, and the like. There is, therefore, substantial interest in being able to develop anti-sense sequences which will be effective as therapeutic agents in the modulation of the formation of mRNA and its expression.

Relevant Literature

Anti-sense nucleic acid sequences have been reported to selectively block translation of a number of mRNAs (Izant and Weintraub, Cell (1984) 36:1007-1015; Izant and Weintraub, Science (1985) 229:345-352; Melton, Proc. Natl. Acad Sci. USA (1985) 82:144-148; Mizuno et al., Proc. Natl. Acad. Sci. USA (1984) 81:1966-1970). Short oligonucleotides have been reported to provide for high selectivity. (Wallace et al., Nucl. Acids Res. (1979) 6:3543-3557; Wallace et al., Nucl. Acids Res. (1981) 9:879-894; Smith, et al., Proc. Natl. Acad. Sci. USA (1986) 83:2787-2791; Szostak, et al., Methods Enzymol. (1979) 68:419-429; Wu et al., Prog. Nucl. Acid Res. and Mol. Biol. (1978) 21:102). The short oligonucleotides are able to rapidly and specifically bind to specific target

sequences. (Itakura and Riggs, Science (1980) 209: 1401; Szostak et al., Methods Enzymol. (1979) 68:419-429; Noyes et al., J. Biol. Chem. (1979) 254:7472-7475; Noyes et al., Proc. Natl Acad. Sci. USA (1979) 76:1770-1774; Agarwal et al., J. Biol. Chem. (1981) 256:1023-1028; Tullis et al., Biochem. Biophys. Res. Comm. (1980) 93:941). See also the use of oligonucleotide probes to detect point mutations. (Orkin et al., J. Clin. Invest. (1983) 71:775; Conner et al., Proc. Natl. Acad. Sci. USA (1983) 80:278; Piratsu et al., New Eng. J. Med. (1983) 309:284-287; Wallace et al., Nucl. Acids. Res. (1981) 9:879-894). The rapidity with which synthetic DNAs hybridize is related to the complexity of the probe. (Wetmur and Davidson, J. Mol. Biol. (1967) 31:349; Wallace et al., Nucl. Acids Res. (1979) 6:3543-3557; Tullis et al., Biochem. Biophys. Res. Comm. (1980) 93:941; Meinkoth and Wahl, Anal. Biochem. (1984) 138:267-284).

Both normal and phosphorous modified oligonucleoides have been reported to selectively block the expression of specific RNAs. Zamecnik and Stephenson, Proc. Natl. Acad. Sci. USA (1978) 75:280-284; Tullis et al., J. Cellular Biochem. Suppl. (1984) 8A:58 (Abstract); Kawasaki, Nucl. Acids Res. (1985) 13:4991; Haeptule et al., Nucl. Acids Res. (1986) 14:1427-1448; Walder et al., Science August 1, 1986; Stephenson and Zamecnik, Proc. Natl. Acad. Sci. USA (1978) 75:285-288 Cornelissen et al., Nucl. Acids Res. (1986) 14:5605-5614; Minshall and Hunt, Nucl. Acids Res. (1986) 14:6433-6451).

Protection from nuclease degradation can be achieved by employing phosphotriesters and methylphosphonates. (Barrett et al., Biochemistry (1974) 13: 4897-4906; Miller et al., Biochemistry (1977) 16:1988-1997; Miller et al., Biochemistry (1981) 20:1873-1880; Jayaraman et al., Proc. Natl. Acad. Sci. USA (1981) 77:1537-1541; Blake et al., Biochemistry (1985)

24:6132-6138; Blake et al., Biochemistry (1985) 24: 6139-6145; Smith et al., Proc. Natl. Acad. Sci. USA (1986) 83:2787-2791; Agris et al., Biochemistry (1986) 25:6268-6275; Miller et al., Nucl. Acids. Res. (1983) 11:6225-6242).

A number of groups have tried to enhance the binding affinity by modifying the nucleotide sequence. (Summerton, J. Theor. Biol. (1978) 78:77-99; Knorre et al., Adv. Enz. Reg. (1984) pp. 277-300; Vlassov et al., Adv. Enz. Reg. (1986) pp. 301-320).

SUMMARY OF THE INVENTION

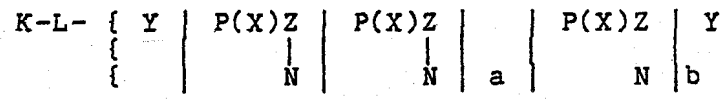
Oligonucleotide conjugates are provided, where a specific sequence of at least eight nucleotides is covalently linked to an ion chelating group and optionally to other groups to enhance transport across the cell membrane. The resulting compositions are found to effectively block the function of a sequence complementary to the oligonucleotide. The compositions find use as agents in vivo and in vitro for modulating intracellular transcription product maturation or expression.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods and compositions are provided for modulating transcriptional maturation or expression by employing oligonucleotide conjugates. The oligonucleotide conjugates have at least two components: an oligonucleotide sequence of at least eight nucleotides; and a chelating agent. In addition, other groups may be present which include a linker joining the chelating agent to the oligonucleotide sequence, a hydrophobic group for enhancing the transport across the membrane, or other moieties to enhance binding affinity, reduce toxicity, enhance solubility, or other characteristics of interest.

The subject compositions will generally have a hybridizing sequence, namely a polynucleotide unit from about 8 to 30, more usually from about 8 to 20, preferably from about 12 to 18 members. The molecular weight will normally be under about 10 kD, usually under about 6 kD, although high molecular weight molecules may be used in special circumstances. The chelating agent may be any one of a large number of chelating agents which are able to chelate metal ions capable of acting as scissile and/or free radical initiating agents, by themselves or in conjunction with other compounds which may be present in the host cell or introduced in the host cell. The chelating agent will be able to chelate one of a variety of metals, such as iron, cobalt, nickel, molybdenum, vanadium, or other metal ion which may be encountered in the cytoplasm of the cell and may serve to initiate the formation of free radicals, resulting in the scission or other modification of the transcription product, preventing its normal function in the host cell.

For the most part, the compositions of the subject invention will have the following formula:



Wherein:

K represents the chelating agent capable of chelating a metal ion, which ion is capable of catalyzing a chemical reaction in the physiological medium of the cytoplasm of a cell, which results in a chemical transformation of mRNA inhibiting expression, particularly degradative modification;

L is a bond or linking unit derived from a polyvalent functional group having at least one atom, which functional group may be of from about 1 to 20 atoms other than hydrogen, comprising carbon, nitrogen,

oxygen, sulfur, and phosphorous, where the linking group may be aliphatic, aromatic, alicyclic, heterocyclic, or combinations thereof; substituted or unsubstituted; generally having from 0 to 10 heteroatoms, usually from 0 to 6 heteroatoms, where the cyclic compounds will usually have from 1 to 2 rings, usually 1 ring, and the aliphatic groups may be branched, straight chained, heterosubstituted or unsubstituted; desirably the linking unit will have a chain of 2 to 20, usually 4 to 16 atoms normally free of linkages capable of enzymatic degradation; L may be joined through Y to the terminal phosphorous or may be joined at any convenient site of the oligonucleotide chain, being linked to P, C, N, O or S of the base (N), saccharide (Z), or group linked to phosphorous (X);

X is usually a pair of electrons, alkyl of from 1 to 3 carbon atoms, chalcogen (oxygen or sulfur), or amino, particularly NH;

Z is a monosaccharide, particularly of 5 to 6 carbon atoms, more particularly of 5 carbon atoms, which may have from 0 to 1 hydroxyl groups replaced by hydrogen, and will usually be substituted by phosphorous at the 2, 3, 5, or 6 positions, particularly at the 3 and 5 positions, and substituted at the one position, by the purine or pyrimidine, where the sugars may include such sugars as ribose, arabinose, xylylose glucose, galactose, deoxy, particularly 2-deoxy, derivatives thereof, etc.;

L' is a linker group which is derived from a polyvalent functional group having at least one atom, and not more than about 60 atoms other than hydrogen, usually not more than about 30 atoms other than hydrogen, having up to about 30 carbon atoms, usually not more than about 20 carbon atoms, and up to about 10 heteroatoms, more usually up to about 6 heteroatoms, particularly chalcogen, nitrogen, phosphorous, etc.;

M is a moiety, particularly imparting amphiphilic properties to the compound, a hydrophobic or amphiphilic moiety which will have a ratio of carbon to heteroatom of at least about 2:1, usually at least about 3:1, frequently up to greater than about 20:1, may include hydrocarbons of at least 6 carbon atoms and not more than about 30 carbon atoms, polyoxy compounds (alkyleneoxy), where the oxygen atoms are joined by from about 2 to 10 carbon atoms, usually 2 to 6 carbon atoms, preferably 2 to 3 carbon atoms, and there will be at least about 6 units and usually not more than about 200 alkyleneoxy units, more usually not more than about 100 units, generally not more than about 60 units;

Y is a bond to L or a terminal group; Y' is a bond to L', linking L' to a terminal phosphorous, or a terminal group; when a terminal group, Y and Y' are oxy, thio, amino or substituted functionalities thereof, e.g., oxyether, alkylamino, etc. or alkyl of up to about 20, usually of up to about 6 carbon atoms, Y and Y' usually not being more than about 20 carbon atoms, more usually being not more than about 10 carbon atoms;

N is any natural or unnatural base (purine or pyrimidine), capable of binding to and hybridizing with a natural purine or pyrimidine, where N may be adenine, cytidine, thymidine, guanidine, uracil, orotidine, inosine, etc.;

a is at least 4, usually at least 5, and not more than about 50, usually not more than about 35;

b and c are each 0 or 1.

The functional groups which find use with the linking groups, L and L', include functionalities such as oxy, non-oxo-carbonyl (carboxy carbonyl), oxo-carbonyl (aldehyde or ketone), the nitrogen or sulfur analogs thereof, e.g. imino, thiono, thio, amidino, etc., disulfide, amino, diazo, hydrazino, oximino, phosphate, phosphono, etc.

The linking group to the hybridizing sequence may be linked through an oxygen or sulfur present on a pyrimidine, purine, sugar or phosphorous group, to a carbon atom of a pyrimidine or purine, or to a phosphorous atom. The links may be ethers to oxygen and sulfur, esters, both organic and inorganic, to oxygen and sulfur, amides, both organic and inorganic, to amines and phosphorous, and alkylamino to amino groups. Esters include carboxylates, e.g. carboxy esters, carbamates, carbonates, etc., and phosphates, phosphonates, etc. Of particular interest is linking at the terminal unit of the hybridizing sequence through a sugar hydroxyl, particularly at the 5'-position.

The phosphorous moiety may include phosphates, phosphoramidates, phosphordiamidate, phosphorothioate, phosphorothionate, phosphorothiolate, phosphoramidothiolate, phosphonates, phosphorimidate, and the like. Where the phosphorous is bound to other than oxygen of the sugar, the sugar will be modified by having an amino or thio functionality at the site of binding, so that both amino and thio sugars may be employed to provide for novel linkages between the phosphorous and the sugar.

K is a chelating agent, having at least 3 heteroatoms, which are oxygen, nitrogen, or sulfur, usually combinations thereof, more usually having about 6 heteroatoms or more, which may serve to chelate a metal ion capable of acting to inactivate, particularly to enhance cleavage, of a nucleic acid. The functionalities may include carbonyl, oxy, thiono, amino, amido, mercapto, thioether, imino, where carbonyl oxygens will normally be separated by at least 2 carbon atoms, usually up to 6 carbon atoms, more usually up to 4 carbon atoms; except for amido, heteroatoms will normally be separated by at least 2 carbon atoms. Conveniently, there will be at least 2 non-oxo-carbonyl groups frequently at least 3 non-oxo-carbonyl groups

and not more than about 6, usually not more than about 5 non-oxo-carbonyl groups. Of particular interest are alkylene diamines and polyalkylene diamines having from 3 to 8, usually 4 to 6 carboxyl groups, usually as carboxymethylene groups, e.g., $R_2N(CH_2)_mN(T)((CH)_nN(J))_x(CH_2)_pNR_2$, wherein R is a carboxyalkylene group of from 2 to 3 carbon atoms or H, at least one R on each N being carboxyalkylene, m, n and p are the same or different and are 2 to 4, usually 2 to 3, and x is 0 to 2.

Illustrative chelating groups include ethylenediaminetetraacetic acid, dipropyleneaminepentaacetic acid, diethylenetriaminepentaacetic acid, 2,3-bis-(2'-acetamidoethyl)succinic acid, porphyrins, phthalocyanins tetraacetic acid, and crown ethers.

A wide variety of linking groups may be employed, depending upon the nature of the terminal nucleotide, the functionality selected for, whether the linking group is present during the synthesis of the oligonucleotide, the functionality present on the hydrophobic moiety and the like. A number of linking groups are commercially available and have found extensive use for linking polyfunctional compounds. The linking groups include: $-OCH_2CH_2NHCO(CH_2)_nCONH-$; $-OCH_2CH_2NH-X-(CH_2)_nNH-$; $-O-P(O)(OH)NHCO(CH_2)_nCOHN-$; $-OCH_2CH_2NHCO\phi S-$; $-NH(CH_2)_nNH$; $-O(CH_2)_nO-$; $-O(CH_2CH_2NH)_m-$; $-NH(CH_2)_nSYN$; $-CO(CH_2)_nCO$; $-SCH_2CH_2CO-$; $-CO\phi NYS-$; $-(NCH_2CH_2)_mCH_2N-$; charged and uncharged homo- and copolymers of amino acids, such as polyglycine, polylysine, polymethionine, etc. usually of about 500 to 2,000 daltons; wherein O is phenyl; X is 2,5-quinondiyl, Y is S-(3-succindoyl) to form succinimidyl, n is usually in the range of 2 to 20, more usually 2 to 12, and m is 1 to 10, usually 1 to 6.

The amphiphilic character imparting or solubility modifying group (M) may be a wide variety of groups, being aliphatic, aromatic, alicyclic, heterocy-

clic, or combinations thereof, substituted or unsubstituted, usually of at least 6, more usually at least 12 and not more than about 1000, usually not more than about 500, more usually not more than about 200 carbon atoms, having not more than about 1 heteroatom per 2 carbon atoms, being charged or uncharged, including alkyl of at least 6 carbon atoms and up to about 30 carbon atoms, usually not more than about 24 carbon atoms, fatty acids of at least about 6 carbon atoms, usually at least about 12 carbon atoms and up to about 24 carbon atoms, glycerides, where the fatty acids will generally range from about 12 to 24 carbon atoms, there being from 1 to 2 fatty acids, usually the 2 or 3 positions or both, aromatic compounds having from 1 to 4 rings, either mono- or polycyclic, fused or unfused, polyalkyleneglycols where the alkylenes are of from 2 to 8, usually of from 2 to 4 carbon atoms, more usually 2 to 3 carbon atoms, there usually being at least about 6 units more usually at least about 10 units, and usually fewer than about 500 units, more usually fewer than about 200 units, preferably fewer than about 100 units, where the alkylene glycols may be homopolymers or copolymers; alkylbenzoyl, where the alkyl group will be at least about 6 carbon atoms, usually at least about 10 carbon atoms, and not more than about 20 carbon atoms; alkyl phosphates or phosphonates, where the alkyl group will be at least 6 carbon atoms, usually at least about 12 carbon atoms and not more than about 24 carbon atoms, usually not more than about 20 carbon atoms, or the like.

The "M" group may be charged or uncharged, preferably being uncharged. Illustrative groups include polyethylene glycol having from about 40 to 50 units, copolymers of ethylene and propylene glycol, laurate esters of polyethylene glycols, triphenylmethyl, naphthylphenylmethyl, palmitate, distearylglyceride didodecylphosphatidyl, cholesteryl, arachidonyl, octadecanyloxy, tetradecylthio, etc.

Functionalities which may be present include oxy, thio, carbonyl, (oxo or non-oxo), cyano, halo, nitro, aliphatic unsaturation, etc.

In designing the nucleic acid sequence, it will be desirable to have a high affinity between the subject composition and the target single stranded nucleic acid sequence. Sequences will preferably be selected having greater than 40% GC content, more preferably greater than 50% and may have 60% or more GC content. For optional selectivity, the melting temperature of the hybrid to be formed should be 5 to 10°C above the ambient temperature at which the hybrid forms, usually the ambient temperature being 37°C in a mammalian host. For mammalian hosts, the melting temperature will generally be chosen to be about 42-50°C. The target sequence should be selected to be relatively free of secondary and tertiary structure. In many mRNA's, an open region will be present in the vicinity of the start codon (AUG).

In preparing the subject compositions, various strategies may be employed, depending upon whether "M" is present, the nature of "M", the nature of the oligonucleotide and the nature of the linking group. Thus, so long as care is taken that the addition of the two different groups, "M" and the chelating group, do not interfere with one another, the groups may be added sequentially.

One technique for providing the chelating agent may be found in Dryer and Dervan, supra. In this technique, a modified nucleoside is employed during the synthesis of the oligonucleotide. Thymidine may be modified at the methyl group by providing for a carboxy alkyl group. The carboxy group may then be further functionalized with an alkylene diamine, and the amino group employed for amide formation with a carboxy containing chelating agent. The modified thymidine may then be employed as a nucleotide reagent in the automated synthesis of the oligonucleotide.

Alternatively, the final nucleotide adduct in the synthesis of the oligonucleotide may be functionalized in a variety of ways which may serve to act as a linking unit to the chelating agent. For example, after removal of the trityl protective group an aminoethanolphosphoramidite is added, as described by the supplier (Applied Biosystems, Foster City, CA) to provide for an available amino group. After deblocking and removing the oligonucleotide chain from the support, the amino group is then available for linking to the chelating agent. Alternatively, the oligonucleotide is phosphorylated employing a polynucleotide kinase, followed by formation of a phosphoramidate using an activating agent, such as 1-methylimidazole or a water soluble carbodiimide, in the presence of an alkylene diamine, providing for an amino functionality (Chu and Orgel, DNA (1985) 4:327-331). A further alternative is to deblock the oligonucleotide while retaining the oligonucleotide on the support, followed by treatment with carbonyldiimidazole. After removal of excess of the carbonyldiimidazole, a diamine may be added to provide an aminoalkylcarbamate (Wachter et al., Nucl. Acids Res. (1986) 14:7985-7994).

Where "M" is to be added, a mercaptan group may be provided as part of the functionalizing agent or separate from the functionalizing agent. The mercaptan group may be part of the linker to the support or may be part of the functionalizing agent of the oligonucleotide, where both the chelating agent and "M" may be bound to the same linking group. Besides mercaptan groups, maleimido groups may be employed, where "M" or the chelating agent may have a mercaptan group to form a thioether.

Various active functionalities can be employed to produce a covalent linkage, such as isocyanates, isothiocyanates, diazo groups, imino chlorides, imino esters, anhydrides, acylhalides, sulfinylhalides,

sulfonyl chlorides, etc. Conditions for carrying out the various reactions and joining non-nucleotide moieties to nucleotide moieties may be found in Chu and Orgel DNA (1985) 4:327-331; Smith et al. Nucl. Acids Res. (1985) 13:2399-2412.

The linking arms, "M", and the chelating moiety may be added at various times, depending upon the particular reaction scheme. For the most part, the chelating agent may be part of a nucleoside and be included in the synthesis of the oligonucleotide or may be added after oligonucleotide formation. "M" will normally be added after oligonucleotide formation.

For the most part, reaction conditions will be mild and will employ polar solvents or combinations of polar and nonpolar solvents. Solvents will vary and include water, acetonitrile, dimethylformamide, diethyl ether, methylene chloride, dimethylsulfoxide, etc. Reaction conditions will be for the most part in the range of about -100-60°C. Usually, after completion of the reaction between components of the conjugate, the resulting product will be subjected to purification.

The manner of purification may vary, depending upon whether the oligonucleotide is bound to a support. For example, where the oligonucleotide is bound to a support, after addition of the linking arm to the oligonucleotide, unreacted chains may be degraded, so as to prevent their contaminating the resulting product. Where the oligonucleotide is no longer bound to the support, whether only reacted with the linking arm or as the conjugate to the chelating agent or as the final product, each of the intermediates or final product may be purified by conventional techniques, such as electrophoresis, solvent extraction, HPLC, chromatography, or the like. The purified product is then ready for use.

The subject products will be selected to have an oligonucleotide sequence complementary to a sequence of interest. The sequence of interest may be present in a prokaryotic or eukaryotic cell, a virus, a normal or neoplastic cell. The sequences may be bacterial sequences, plasmid sequences, viral sequences, chromosomal sequences, mitochondrial sequences, plastid sequences, etc. The sequences may involve open reading frames for coding proteins, ribosomal RNA, snRNA, hnRNA, introns, untranslated 5'- and 3'-sequences flanking open reading frames, etc. The subject sequences may therefore be involved in inhibiting the availability of an RNA transcript, inhibiting expression of a particular protein, enhancing the expression of a particular protein by inhibiting the expression of a repressor, reducing proliferation of viruses or neoplastic cells, etc.

The subject conjugates may be used in culture or in vivo for modifying the phenotype of cells, limiting the proliferation of pathogens such as viruses, bacteria, protista, mycoplasma, or the like, or inducing morbidity in neoplastic cells or specific classes of normal cells. Thus, one can use the subject compositions in therapy, by administering to a host subject in a diseased state, one or more of the subject compositions to inhibit the transcription and/or expression of the native genes of a cell. The subject compositions may be used for protection of a mammalian host from a variety of pathogens, e.g., enterotoxigenic bacteria, Pneumococcus, Neisseria, etc.; protists, such as Giardia, Entamaeba, etc.; neoplastic cells, such as lymphoma, leukemia, carcinoma, sarcoma etc.; specific B-cells, specific T-cells, such as helper cells, supressor cells, CTL, NK, etc.

The subject compositions will be selected so as to be capable of inactivating sequences of interest, particularly mRNA, or in some circumstances the subject

composition can be used with other nucleic acid moieties, e.g., tRNA, snRNA, DNA, e.g., plasmids, viruses, etc. Thus, the subject compositions may bind to mRNA and provide for cleavage of the mRNA, so as to prevent the expression of a product. By employing sequences which are relatively inert to degradation, the lifetime of the chelate conjugate may be substantially extended in the host cell, so as to have a relatively high kill ratio per sequence.

The subject sequences may be complementary to such sequences as sequences expressing growth factors, lymphokines, immunoglobulins, T-cell receptor sites, MHC antigens, DNA or RNA polymerases, antibiotic resistance, multiple drug resistance (mdr), genes involved with metabolic processes, in the formation of amino acids, nucleic acids, or the like, DHFR, etc. as well as introns or flanking sequences associated with the open reading frames.

The subject compositions may be administered to a host in a wide variety of ways, depending upon whether the compositions are used in vitro or in vivo. In vitro, the compositions may be introduced into the nutrient medium, so as to modulate expression of a particular gene by transferring across the membrane into the cell interior such as the cytoplasm and nucleus. The subject compositions may find particular use in protecting mammalian cells in culture from mycoplasma, for modifying phenotype for research purposes, for evaluating the effect of variation of expression on various metabolic processes, e.g., production of particular products, variation in product distribution, or the like. While no particular additives are necessary for transport of the subject compositions intracellularly, the subject compositions may be modified by being encapsulated in liposomes or other vesicle, may be used in conjunction with permeabilizing agents, e.g., non-ionic detergents, Sendai virus, etc.

For in vivo administration, depending upon its particular purpose, the subject compositions may be administered in a variety of ways, such as injection, infusion, tablet, etc., so that the compositions may be taken orally, parenterally, intravascularly, intraperitoneally, subcutaneously, intralesionally, or the like. The compositions may be formulated in a variety of ways, being dispersed in various physiologically acceptable media, such as deionized water, water, phosphate buffered saline, ethanol, aqueous ethanol, formulated in the lumen of vesicles, microencapsulated, etc.

Because of the wide variety of applications and manners of administration, no particular composition can be suggested. Rather, as to each indication, the subject compositions may be tested in conventional ways and the appropriate concentrations determined empirically. Other additives may be included, such as stabilizers, buffers, additional drugs, detergents, etc. These additives are conventional, and would generally be present in less than about 5 wt%, usually less than 1 wt%, being present in an effective dosage, as appropriate. For fillers or excipients, these may be as high as 99.9% of the composition, depending upon the amount of active material necessary.

The following examples are presented by way of illustration not by way of limitation.

EXAMPLE 1

Synthesis of Diethylenetriamine pentacetic acid (DTPA) Conjugated Oligomers

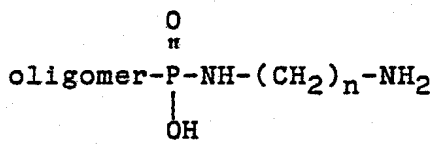
Chemical Synthesis of DNA. The chemical synthesis of DNA was carried out using slight modifications of the conventional phosphoramidite methods on an Applied Biosystems (Model 381) DNA synthesizer. The method used is a modification of the technique described by Caruthers and coworkers (Beaucage and Caruthers, 1984, European Patent Application 82/102570).

In this technique, nucleoside phosphoramidites dissolved in anydrous acetonitrile are mixed with tetrazole and sequentially coupled to the 5' hydroxy terminal nucleotide of the growing DNA chain bound to controlled pore glass (CPG) supports via a succinate spacer (Matteucci and Caruthers, Tetrahedron Letters (1980) 21:719-722). Nucleoside addition is followed by capping of unreacted 5' hydroxyls with acetic anhydride, iodine oxidation, and 5' detritylation in trichloroacetic acid-methylene chloride. The resin bound oligomer is then dried by extensive washing in anhydrous acetonitrile and the process repeated. Normal cycle times using this procedure are 12 minutes with condensation efficiencies of >98% (as judged by trityl release).

Chemical Synthesis of Amine Linker Arm Containing DNA Oligonucleotides Bound to CPG Glass Beads. The completed fully blocked DNA chains can subsequently be modified to contain a 5' amino linker arm while still attached to the CPG support. Several methods have been used to accomplish this.

Aminolink Procedure for 5' Amine Linker Arm Attachment. At the end of the synthesis, trityl is removed from the product oligonucleotide chains and an aminoethanolphosphoramidite is added to the 5' hydroxyl using the Aminolink procedure developed and marketed by Applied Biosystems (Foster City, CA). The resin bound oligonucleotide is then deblocked and released from the column using a method appropriate to the type of phosphate linkage present. For normal phosphodiesters, hydrolysis in concentrated ammonia is appropriate. For DNA triesters and methylphosphonates, ethylene diamine (EDA) phenol deblocking followed by ammonia or EDA: ethanol release is appropriate. Results indicate that all of the cyanoethyl-phosphorus and aryl-amide base blocking groups are removed under these conditions.

Addition of Amine Containing Linker Arms Using Phosphoramidate Linkage. One alternative is the use of the technique of Chu and Orgel, Proc. Natl. Acad. Sci. USA (1986) 82:963-967 to add linker arms to the 5' end of any oligonucleotide. In this instance, the oligonucleotide is first phosphorylated using polynucleotide kinase. After purification by polyacrylamide gel electrophoresis, the product DNA containing a free 5'-hydroxyl is phosphorylated with the forward reaction of T4 polynucleotide kinase according to Chu and Orgel, supra (1986). Phosphorylated oligomers are separated from unreacted ATP using a C-18 reverse phase column (Waters SEP-PAK) according to the direction of the manufacturer. The phosphorylated oligomer is then treated with 0.1M 1-methyl imidazole, 0.1M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 0.1M alkyl diamine (e.g., hexane diamine), pH 7, under conventional conditions in an aqueous medium to form the desired phosphoramidate containing a free amine with the following structure



Addition of An Amine Linker Arm Using Carbonyl Diimidazole (CDI). A third alternative to the addition of an amine linker arm to the 5' end of any oligonucleotide is to use carbonyl-bis (imidazole). In this technique, the CPG bound, base blocked oligomer (trityl off) is first treated with CDI (50 mg/ml) in dry acetonitrile for 30 to 45 minutes to form the imidazole carboxylic acid ester. The excess CDI is then washed off with acetonitrile and water and the diamine of choice added to the column generally in a mixture of acetonitrile or dioxane and water. The diamine forms a

stable carbamate linkage after a brief incubation. The oligomer can then be deblocked and released from the column under conditions appropriate to the type of phosphate linkage present.

Attachment of the Cleavage Unit DTPA. Once the amine terminated oligomer is deblocked and characterized, the cleavage unit was added using the following method. Ten units of the oligomer were dissolved in 100 µl dimethylformamide containing 0.1 M DTPA (bis-anhydride). The mixture was incubated for 1-2 hours at room temperature, and the excess DTPA removed by gel filtration and concentration on Centricon C10 membranes. The final product was dried in vacuo and dissolved to a final concentration of 1 mM in water (based on its optical density at 260nM) and stored frozen. This solution was stable for at least 1 month. The compound was homogeneous as judged by polyacrylamide gel electrophoresis.

EXAMPLE 2

Synthesis of DTPA Derivatives of Normal DNAs Using Imidazole Activated Carboxylic Acid Esters and Long Chain Aminoalkanes

In this example, a 20 nucleotide DNA complementary to the initiation region of mouse beta globin mRNA was synthesized according to the method given in EXAMPLE 1. After synthesis, the product material was retained on the synthesis support with trityl removed from the 5' end of the molecule. The solid material was then thoroughly washed with anhydrous acetonitrile and blown dry under a stream of dry argon. Using a plastic syringe, 1 cc of 0.3M carbonyldiimidazole dissolved in anhydrous acetonitrile was pushed slowly through the synthesis column containing the support bound oligomer over a period of 45 minutes. The 5' carbonylimidazole activated oligomer on the column was then washed free of excess reagent with 15 ml of aceto-

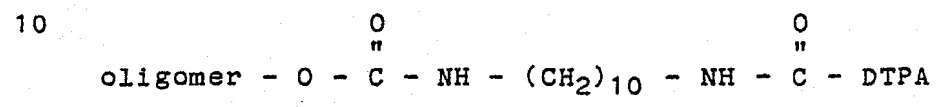
nitrile and then treated with 0.2 M decanediamine in acetonitrile:water (10:1) for 30 minutes.

The material on the column was washed free of unreacted decane-diamine with acetonitrile and water, and then eluted from the column in concentrated ammonium hydroxide. After removal from the column, the ammonium hydroxide solution containing the oligomer conjugate was placed in a sealed vial and incubated 5 hours at 55°C.

The product is then lyophilized several times from 50% aqueous ethanol and purified via reversed phase HPLC C-8 silica columns eluting 5 to 50% acetonitrile/25 mM ammonium acetate, pH 6.8 in a linear gradient. If required, the material was further purified by ion-exchange HPLC on Nucleogen DEAE 60-7 eluting 20% acetonitrile/25 mM ammonium acetate, pH 6.5. The recovered product is then characterized by gel electrophoresis in 15% polyacrylamide gels carried out as described by Maxam and Gilbert (Meth. Enzymol. (1980) 68:499-560). Oligonucleotides in finished gels are visualized using Stains-all.

As a further check on the material, the presence of a primary amine can be determined by two methods. First, reaction with fluorescamine produced a fluorescent product characteristic of the presence of a primary amine while no fluorescence is observed with similarly treated control oligomers of the same type, but lacking the amine linker. Second, the decane-conjugate was dissolved in 100 µl 0.1 M sodium bicarbonate to which was added 1 mg of fluorescein isothiocyanate. After 1 hour of incubation, the unreacted FITC was removed by gel filtration chromatography on Sephadex G-25 spun columns. The product was then analyzed by polyacrylamide gel electrophoresis as described above, and the fluorescent band product visualized under UV illumination. A single fluorescent band is observed which corresponds to the oligomer visualized by subsequent staining with Stains-all.

The product of this reaction is an aminoalkyl carbamate coupled to the 5' end of the oligonucleotide. The alkylcarbamate is stable to moderate exposure to concentrated base. The free amino group distal to the carbamate linkage is available for subsequent derivation which can be accomplished according to the method given in EXAMPLE 1. The structure of the final conjugate synthesized by this method is illustrated as:



EXAMPLE 3

Synthesis of DTPA Derivatives of DNAs Using Imidazole Activated Carboxylic Acid Esters and a Poly-D-lysine Linker

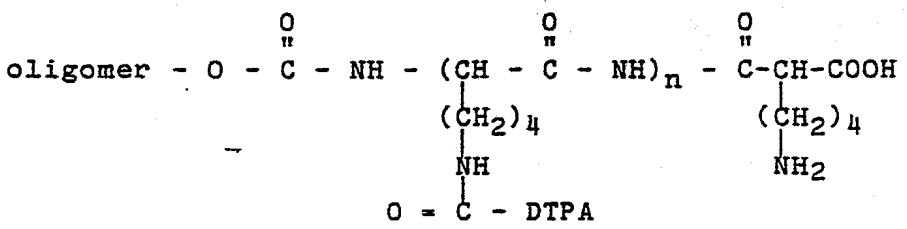
In this example, a 25 nucleotide DNA complementary to the initiation region of rabbit beta globin mRNA was synthesized according to the method given in EXAMPLE 1. After synthesis, the CPG support containing the oligomer was treated with 80% acetic acid for 30 minutes to remove trityl from the 5' end of the molecule. The solid material was then thoroughly washed with anhydrous acetonitrile and blown dry under a stream of dry argon and treated with 0.3M CDI as in EXAMPLE 4. The 5' CDI activated oligomer on the column was then washed free of excess reagent with 15 ml of acetonitrile and then treated with 0.2 M poly-D-lysine (MW+1,000) dissolved in 50% acetonitrile containing 0.1 M sodium phosphate, pH 8 for 16 hours at room temperature.

The material on the column was washed free of salts and unreacted polylysine with water and acetonitrile and then eluted from the column in concentrated ammonium hydroxide. After removal from the column, the ammonium hydroxide solution containing the oligomer

conjugate was incubated for 5 hours at 55°C in a sealed glass vial. The product was then lyophilized several times from 50% aqueous ethanol and purified via gel filtration chromatography on TSK G4000SW in 100 mM Tris buffer, pH 7.5. The presence of a primary amine was determined by reaction with fluorescamine. No fluorescence was observed with control oligomers lacking the polyamine linker.

The polyamine conjungate cannot easily be characterized by gel electrophoresis since it is electrostatically and molecularly polydisperse. In order to render the polyamine conjugate negatively charged, the complex was reacted with FITC to label the molecule and to neutralize the positive charges on the amines. This was accomplished by dissolving a portion of the material in 100 µl 0.1 M sodium bicarbonate to which was added 1 mg of fluoresceinisothiocyanate. After 1 hour of incubation, the unreacted FITC was removed by gel filtration chromatography on Sephadex G-25 spun columns (Maniatis et al., Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Lab., Cold Spring Harbor, New York (1982)). The product was then analyzed by polyacrylamide gel electrophoresis carried out as described by Maxam and Gilbert (Meth. Enzymol. (1980) 68:499-560) and the fluorescent band product visualized under UV illumination. A single broad fluorescent band is observed which corresponds to the DNA visualized by Stains-all.

The structure of this conjugate may be illustrated as having the general formula:



By varying the reaction excess of the DTPA or the molecular size of polylysine used, it is possible to construct conjugates with varying degrees of substitution, size and charge. The ability to vary these properties of the complex make it possible to design the use of the compound in various applications.

EXAMPLE 4

Synthesis of DTPA Derivatives of DNA Methylphosphonates

The chemical synthesis of DNA methylphophonates may be carried out using a modification of the phosphochloridite method of Letsinger (Letsinger et al., J. Am. Chem. Soc. (1975) 97:3278; Letsinger and Lunsford, J. Am. Chem. Soc. (1976) 98:3655-3661; Tanaka and Letsinger, Nucl. Acids Res. (1982) 10:3249). However, the preferred method and the one used in this example uses methyl phosphonamidites (Applied Biosystems, Foster City, CA). The method for performing the synthesis uses exactly the same steps and reaction times as in conventional DNA synthesis, with the exception that THF rather than acetonitrile is used to dissolve the phosphonamidites due to their reduced solubility in the latter. Normal cycle times using this procedure are 15 minutes with condensation efficiencies of >94% (as judged by trityl release). The ultimate base may be added as the cyanoethyl phosphotriester which yields, upon cleavage in base, a 5' terminal phosphodiester. This step makes it possible to radiolabel the oligonucleotide, purify and sequence the product using gel electrophoresis at intermediate stages of preparation (Narang et al, Can. J. Biochem. (1975) 53:342-394; Miller et al., Nucl. Acids Res. (1983) 11:6225-6242).

An amine terminated linker arm is then added as follows. Trityl is removed as before and the resin

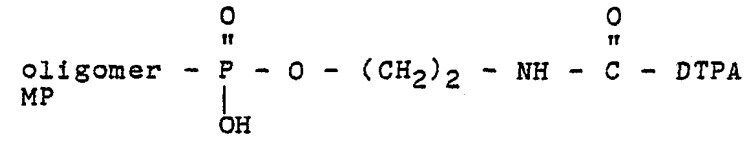
treated with 0.2 M Aminolink (Applied Biosystems, Foster City, CA) dissolved in dry acetonitrile containing 0.2M dimethylaminopyrodine for 5 minutes. The linker arm oligonucleotide is then oxidized in iodine and washed in acetonitrile as above. Capping with acetic anhydride is not performed since any deblocked primary amine would be modified to the base stable acetamide and thus unavailable for further reaction.

At the end of the synthesis, the amine terminated linker arm methylphosphonate oligomer is base deblocked as follows. The resin containing the DNA is removed from the column and placed in a water jacketed column and incubated in 1-2 ml phenol:ethylene diamine (4:1) for 10 hours at 40°C. At the end of the incubation in phenol:ethylene diamine, the resin is washed free of the phenol reagent and released base protecting groups using methanol, water, methanol and methylene chloride in succession. After drying in a stream of nitrogen, the intact, base-deblocked chains are cleaved from the support using EDA:ethanol (1:1) or brief treatment with room temperature ammonium hydroxide.

Purification of the amine terminated DNA methylphosphonate is then performed as follows. The material is first lyophilized several times from 50% aqueous ethanol and purified via reversed phase HPLC C-8 silica columns eluting 5 to 50% acetonitrile/25 mM ammonium acetate, pH 6.8 in a linear gradient. Amine containing fractions as determined by fluorescamine reactivity are pooled and the product recovered by drying in vacuo and further purified by ion-exchange HPLC on Nucleogen DEAE 60-7 eluting 20% aceonitrile/25 mM ammonium acetate, pH 6.5.

The purified product is then converted to the DTPA derivative as in EXAMPLE 1. Purification of the complex is then effected as previously described. Alternatively, unbound oligonucleotide is removed by gel filtration on Sephadex G-100 or HPGFC on TSK G4000SW eluting 10 mM Tris, pH 7.5.

The structure of the final product of this procedure is illustrated as:



EXAMPLE 5

Synthesis of DTPA Derivatives of DNA Ethyltriesters Using the Phosphoramidite Approach

The synthesis of the title compound triesters is performed according to the method of Zon and coworkers (Gallo et al., Nucl. Acids Res. (1986) 14:7405; Summers et al., Nucl. Acids Res. (1986) 14:7421-7436). The method of synthesis is similar to that which is used for in situ production with ethyl triesters as described by Letsinger (Letsinger et al., J. Am. Chem. Soc. (1975) 97:3278; Letsinger and Lunsford, J. Am. Chem. Soc. (1976) 98:3655-3661; Tanaka and Letsinger, Nucl. Acids Res. (1982) 10:3249). In brief, fully blocked dimethoxytrityl nucleosides are derived by repeated lyophilization from benzene, dissolved in anhydrous acetonitrile/2,6-lutidine (8:2) and added dropwise to a stirred solution of chloro diisopropyl-amino ethoxyphosphine in the same solvent at -70°C. The product is recovered by aqueous extraction, in vacuo drying and silica gel chromatography.

The chemical synthesis of DNA can be carried out using slight modifications of the conventional phosphoramidite methods. In this technique, nucleoside phosphoramidites dissolved in anhydrous acetonitrile are mixed with tetrazole and sequentially coupled to the 5' hydroxy terminal nucleoside bound to CPG. Nucleoside addition is followed by capping of unreacted 5' hydroxyls with acetic anhydride, iodine oxidation, and 5' detritylation in trichloroacetic acid-methylene

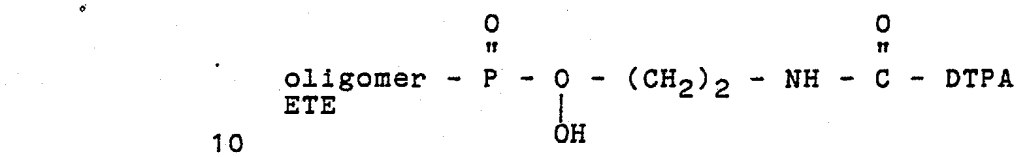
chloride. The resin bound oligomer is then dried by extensive washing in anhydrous acetonitrile and the process repeated. Normal cycle times using this procedure are 17 minutes with condensation efficiencies of >96% (as judged by trityl release). The terminal residue is conventionally added as a diester in order to facilitate radiolabeling and purification. The 5' terminal trityl group is left if HPLC purification is desired, but generally the 5' terminal trityl is removed and the aminolink procedure described in EXAMPLE 1 is used.

At the end of the synthesis, the fully blocked product is base-deblocked as follows. The resin containing the fully protected DNA is removed from the column and placed in a water jacketed chromatography column. The resin is then incubated in 1-2 ml phenol:ethylene diamine (4:1) for 10 hours at 40°C. At the end of the incubation in phenol:ethylene diamine, the resin is washed free of the phenol reagent and released base protecting groups using methanol, water, methanol and methylene chloride. After drying in a stream of nitrogen, the intact, base-deblocked chains are cleaved from the support using EDA:ethanol (1:1) or brief treatment with room temperature ammonium hydroxide.

Purification of the aminolink DNA ethyltriester product is then performed as follows. The material is first lyophilized several times from 50% aqueous ethanol and purified via reversed phase HPLC C-8 silica columns eluting 5 to 50% acetonitrile/25 mM ammonium acetate, pH 6.8 in a linear gradient. Amine containing fractions as determined by fluorescamine reactivity are pooled and the product recovered by drying <u>in vacuo</u> and further purified by ion-exchange HPLC on Nucleogen DEAE 60-7 eluting 20% acetonitrile/25 mM ammonium acetate, pH 6.5.

The product oligonucleotide is then suitable for coupling to DTPA and purification by the techniques previously described.

The structure of the final product of this procedure is illustrated as:



EXAMPLE 6

Synthesis of DTPA Derivatives of DNA Alkyl and Aryltriesters Using the Phosphate Triester Approach

A preferred method for the production of the oligonucleotide triesters of variable alkane chain length is via conventional phosphate triester chemistry to synthesize the desired sequences as the p-chlorophenyl phosphate triesters. Upon completion of the synthesis, the fully protected oligonucleotide chlorophenyltriesters bound to the synthesis support are subjected to ester exchange in the presence of tetrabutylammonium fluoride and the desired alcohol. This basic method for the construction of DNA oligonucleotides is classical DNA synthesis chemistry and presents no problems. The essential chemistry is well described (Gait, Oligonucleotide Synthesis: A Practical Approach IRL Press, Washington, D.C. (1984)) and can be used with little modification. An alternative phosphite based chemistry which is much more rapid and gives equivalent yields is set forth below.

The chemical synthesis of DNA p- or o-chlorophenyl phosphotriesters was carried out using a modification of the phosphochloridite method of Letsinger (Letsinger et al., J. Am. Chem. Soc. (1975) 97:3278; Letsinger and Lunsford, J. Am. Chem. Soc.

(1976) 98:3655-3661; Tanaka and Letsinger, Nucl. Acids Res. (1982) 10:3249). A programmable, automated DNA synthesizer used for phosphomonochloridite based syntheses (Alvarado-Urbina et al., Science (1981) 214:270-273.

Fully blocked and carefully dried nucleosides dissolved in anhydrous acetonitrile, 2,6-lutidine and activated in situ with chlorophenoxydichlorophosphine are sequentially added to the 5' hydroxy terminal nucleotide of the growing DNA chain bound to controlled pore glass supports via a succinate spacer (Matteucci and Caruthers, Tetrahedron Lett. (1980) 21:719-722). Derivatized glass supports, fully block nucleosides and other synthesis reagents are commercially available through Applied Biosystems (San Francisco, CA) or American Bionuclear (Emeryville, CA). Nucleoside addition is followed by capping of unreacted 5' hydroxyls with acetic anhydride, iodine oxidation, and 5' detritylation in trichloroacetic acid-methylene chloride.

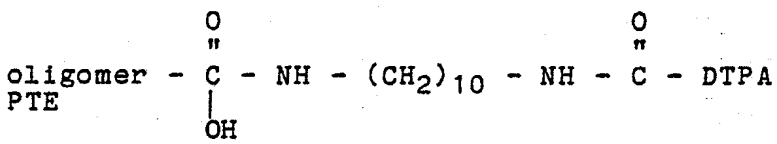
The resin bound oligomer chlorophenyltriester is then dried by extensive washing in anhydrous acetonitrile and the process repeated. Normal cycle times using this procedure are 13 minutes with condensation efficiencies of >92% (as judged by trityl release). The ultimate base may be added as a methyl phosphotriester which yields, upon cleavage in base, a 5' terminal phosphodiester. This step makes it possible to radiolabel the oligonucleotide and to purify and sequence the product using gel electrophoresis (Narang et al., Can. J. Biochem. (1975) 53:392-394; Miller et al., Nucl. Acids Res. (1983) 1:6225-6242).

The fully blocked material bound to the synthesis support is then subjected to ester exchange in the presence of tetrabutylammonium fluoride (TBAF) and the desired alcohol. This method yields rapid and quantitative alcohol exchange. The reaction is com-

plete within 20 minutes for most aryl and alkyl alcohols which are capable of forming stable products. With any alcohol, the presence of trace amounts of water can affect the overall yield. Thus, care must be taken to used anhydrous alcohols at this step.

In this example, anhydrous n-propanol is used to dissolve TBAF to a final concentration of 0.2 M. The solution is then percolated slowly over the resin containing the oligomer chlorophenyl triester and allowed to react for about 1 hour at room temperature. The resin is then washed with methanol and acetonitrile and dried under a stream of dry argon. Amine linker arm addition, deblocking and purification are then effected as in EXAMPLE 2. DTPA conjugation is then performed as in EXAMPLE 1. The final yield of conjugate is about 10% of the starting equivalents of nucleoside resin used.

The structure of the final product is illustrated as:



EXAMPLE 7

Effect of DTPA Conjugates on the Synthesis of Hemoglobin in Mouse MEL Cells

The effectiveness of oligomer DTPA conjugate mediated HART (Paterson et al., Proc. Natl. Acad. Sci. USA (1977) 74:4370; Hastie and Held, Proc. Natl. Acad. Sci. USA (1978) 75:1217-1221) was determined in cultured cells incubated in the presence or absence of the oligomer. The cells used were Friend murine erythroleukemia (MEL) cells which can be induced to synthesize hemoglobin by a variety of agents including DMSO and butyric acid (cf. Gusella and Houseman, Cell

(1976) 8:263-269). Friend leukemia cells were grown in culture using well known techniques in a CO_2 incubator. Hemoglobin synthesis was induced using 1.5% DMSO.

Induced cells expressing hemoglobin were visualized by benzidine treatment which stains globin producing cells blue (Leder et al., Science (1975) 190:893). Cells were exposed to selected oligonucleotides and DTPA conjugate at concentrations ranging from 0.1 μM to 50 μM during a 4- to 5-day induction period. Controls included mock-treated cells and cells treated with unmodified oligomers of the same sequence. Treated cells were scored for globin production based on staining intensity and the results compared to controls. Cell death or damage due to treatment was scored by Trypan blue exclusion.

The following Table (Table I) indicates the specific sequences synthesized.

TABLE I

DNA SEQUENCES SYNTHESIZED AND CONJUGATED FOR USE IN CELL CULTURE EXPERIMENTS

Probes Synthesized Antisense to Mouse Beta-globin mRNA	% GC	Sequence (3' to 5')
MBG 15 methylphosphonate	60%	g tac cac gtg gac tG
MBG 15 methylphosphonate-C_2amine	60%	g tac cac gtg gac tGp-O-$(CH_2)_2$-NH_2
MBG 15 methylphosphonate DTPA conjugate	60%	g tac cac gtg gac tGp-O-$(CH_2)_2$ -NH-C(O)-DTPA
MBG 20 antisense	55%	G TAC CAC GTG CAC TGA CTA C
MBG 20 antisense C_2-amine	55%	G TAC CAC GTG CAC TGA CTA Cp-O-$(CH_2)_2$-NH_2
MBG 20 antisense C_6-amine	55%	G TAC CAC GTG GAC TGA CTA C-O-(CO)-NH-$(CH_2)_6$-NH_2
MBG 20 antisense C_2-amine DTPA conjugate	55%	G TAC CAC GTG CAC TGA CTA Cp-O-$(CH_2)_2$-NH-C(O)-DTPA

a) Lower case letters represent nucleosides coupled to the 3' adjacent nucleoside via a methylphosphonate linkage. Upper case letters represent 3' adjacent normal phosphodiester linkage. C_2 derivatives are formed from the condensation of ethanolamine with a 5' terminal phosphate via an ester linkage. C_6 and C_{10} derivatives are the corresponding diamines coupled via an alkyl carbamate linkage to the 5' terminal hydroxyl. DTPA represents diethylenetriamine pentaacetic acid.

The data in the following Table (Table II) show that DTPA conjugated oligomers were approximately 500 times more effective than control oligomers with or without the amine linker attached. Significant cytotoxicity was observed only at concentrations above 10 μM, about 100 times the minimum dose for a significant effect.

TABLE II

The Effect of Cleaver Conjugate Oligonucleotides in Preventing the Synthesis of Hemoglobin in Cultured Cells

Treatment (1)	Conc.	% Viable Cells	% Inhibition Globin$^+$ Cells
Experiment #1 - Effect Compared to Other Constructs.			
DMSO Control		46%	0%
MBG-20 Antisense	50 µM	50%	41%
MBG-20-C_2amine	50 µM	61%	41%
MBG-20-DTPA Conjugate	50 µM	0%	100%
Experiment #2 - Concentration Dependence of DTPA Conjugate			
DMSO Control	1.5%	65%	0%
Solvent Control		n.d	0%
EDTA Control	1 µM	65%	0%
MBG-20-DTPA Conjugate	1 µM	50%	98%
		57%	96%
	500 nM	34%	95%
		14%	96%
	300 nM	65%	84%
		65%	84%
	200 nM	78%	58%
	100 nM	69%	56%
		76%	60%
	10 nm	67%	47%
		74%	61%
	1 nM	70%	41%
	0.1 nM	72%	2%
	none	nd	0%

Table II
(continued)

Experiment #3 - Effect of Methylphosphonate DTPA Conjugate

Treatment ([1])	Conc.	% Viable Cells	% Inhibition
DMSO Control		50%	0%
MBG 15 methyl-phosphonate DTPA Conjugate	100 nM	50%	48%
	10 nM	51%	43%
	1 nM	54%	37%
	0.1 nM	54%	37%

([1]) C_2 derivatives are formed from the condensation of ethanolamine with a 5' terminal phsophate via an ester linkage. DTPA represents diethylenetriamine pentaacetic acid.

It is evident from the above results, that conjugates of a chelating agent and an oligonucleotide sequence may be used to preferentially inhibit the expression of a sequence in a viable cell. In this manner, cells can be modified in a variety of ways, so as to change the phenotype or to selectively kill cells. The conjugate is stable and does not require that a metal be non-covalently bound to the chelating agent prior to use in order to achieve effectiveness. In addition, the subject compositions can be used *in vivo* or *in vitro*, allowing for selection of cells, enhancing activity of particular cells, reducing activity of particular cells, or permitting selection of a particular class of cells. A variety of conjugates can be produced, which will have long half lives, so as to be able to provide for destruction of a large number of RNA sequences for each molecule conjugate.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention

pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of inhibiting the utilization of RNA in a cellular process, said method comprising:

contacting cells with a composition comprising (1) a hybridizing sequence capable of hybridizing with an RNA sequence; joined to (2) a metal-ion free chelating agent capable of chelating a metal ion, said metal ion capable of initiating an intracellular reaction resulting in the inactivation of said RNA sequence,

whereby said composition enters said cell and inactivates said RNA sequence.

2. A method according to Claim 1, wherein said hybridizing sequence is at least 50 number percent G + C.

3. A method according to Claim 1, wherein said chelating agent is a polyamino-polycarboxylic acid chelating agent.

4. A method according to Claim 3, wherein said chelating agent is an alkylenepolyamine polycarboxymethylene chelating agent.

5. A method according to Claim 1, wherein said chelating agent is linked to said hybridizing sequence through a linking chain of from 4 to 16 atoms in the chain.

6. A method according to Claim 1, wherein said cell is part of a mammalian host.

7. A method according to Claim 1, wherein said composition further comprises a moiety imparting amphiphilic properties to said composition, said moiety comprising at least 6 carbon atoms and hydrophobic or amphiphilic.

8. A method of inhibiting the utilization of mRNA in a cellular process, said method comprising:

contacting cells with a composition comprising (1) a hybridizing sequence capable of hybridizing with an mRNA sequence in said cells; joined to (2) a metal-ion free polyamino-polycarboxylic acid chelating agent capable of chelating a metal ion, said metal ion capable of initiating an intracellular reaction resulting in the inactivation of said RNA sequence,

whereby said composition enters said cell and inactivates said mRNA sequence.

9. A method according to Claim 8, wherein said chelating agent is diethylene triamine pentaacetic acid.

10. A method according to Claim 9, wherein said chelating agent is joined to said hybridizing sequence through a linking chain of from 4 to 16 atoms in the chain and through a amide functionality.

11. A method according to Claim 8, wherein said hybridizing sequence of is a polyphosphate.

12. A method according to Claim 8, wherein said hybridizing sequence is a polyphosphonate.

13. A method according to Claim 8, wherein said hybridizing sequence has from about 8 to 30 units.

14. A method according to Claim 8, wherein said composition further comprises a moiety imparting amphiphilic properties to said composition, said moiety comprising at least 6 carbon atoms and hydrophobic or amphiphilic.

15. A composition comprising a hybridizing sequence comprising a total of from about 8 to 30 nucleosides and capable of hybridizing to an RNA sequence

covalently joined through a linking chain of from about 4 to 16 atoms in the chain to a polyamino-polycarboxylic acid chelating agent, said linking arm bonded to a sugar of said nucleoside.

16. A composition according to Claim 15, wherein said linking arm is bonded to said sugar through an ester linkage.

17. A composition according to Claim 16, wherein said ester linkage is to a 5' hydroxyl of a sugar of the terminal unit of said hybridididizing sequence.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/04430

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12N 05/00; A61K 31/00

U.S. Cl. 435/240.2, 514/44

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/6,41,91,240.2; 935/78; 514/44

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Computer searched CAS (1967-1989), Biosis (1969-1989), APS (1975-1989). Terms: chelate, diethylene triamine pentaacidic acid, transport

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4707352 (Stavrianopoulos) 17 November 1987. See entire document.	1-17
Y	US, A, 4508625 (Graham) 02 April 1985. See entire document.	1-17
Y	Suzuki et al, An Introduction to Genetic Analysis, published 1986, by W.H. Freeman and Company (New York), see pages 191-193.	2

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06 February 1989	19APR 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Lori Y. Beardell Lori Y. Beardell

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